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(54) Title: VIABILITY PROBES FOR ISOLATION, IDENTIFICATION AND/OR ANALYSIS OF CELLS

(57) Abstract

The present invention provides a viability probe which can be used independently or is useful for inclusion in a monoclonal antibody panel for the analysis of solid or non-adherent tumors.

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VIABILITY PROBES FOR ISOLATION, IDENTIFICATION AND/OR ANALYSIS OF CELLS

Technical Field

The present invention provides viability probes which can be use independently or are useful for inclusion in a monoclonal antibody panel for the analysis of solid or non-adherent tumors.

Background Art

Non-viable, permeabilized cells constitute a significant source of interference in flow cytometric analysis. This interference results primarily from differential antigen expression (13), biochemical changes (e.g., increased autofluorescence) (24), and the nonspecific uptake and binding of probes due to membrane permeabilization accompanying cell death (6,15,20,22). Accurate analysis of the viable cells, therefore, requires exclusion of the non-viable cells (20).

The two best characterized processes of cell death are necrosis and apoptosis (1,3,5,9,13,19). Necrosis is typically induced by extracellular conditions, such as hypoxia or cytotoxic agents, and is characterized by a sequence of metabolic collapse, cell swelling, lysis, and inflammation of surrounding tissue (1,5,7,9,23). The more common phenomenon of apoptosis (1,10) is associated with programmed cell death, and is characterized by a sequence of cell shrinkage, chromatin condensation and cleavage, and finally, membrane permeabilization (1,3,5,7,9,10,13). As dying cells permeabilize, changes in their morphology and refractive index significantly alter the cells' light scatter characteristics, and the loss of membrane integrity permits uptake of normally impermeant probes.

The most widely used flow cytometric dead cell discrimination techniques can be divided into three main categories: (1) physical separation methods, (2) differential light scatter, and (3) differential staining by fluorescent probes. These techniques are largely

measures of membrane permeabilization, and are based on the equation of this phenomenon with cell death (6,13).

It is apparent, therefore, that treatment of samples with permeabilizing agents, as is often necessary for sample preservation, intracellular labelling, or biohazard control, presents a significant challenge to the discrimination of naturally permeabilized dead cells from cells that were alive prior to artificial permeabilization. Such agents can drastically alter cellular morphology and refractive index, resulting in homogenization of the light scatter characteristics of live and dead cells, as well as cells of different types. Moreover, permeabilizing agents allow all cells, including those that were live prior to permeabilization, to take up any probe present in excess in the solution, or that leaks back out of previously stained dead cells. Consequently, fixation and permeabilization generally preclude the use of the two most common fresh prep techniques for dead cell discrimination, i.e., electronic gating on the basis of light scatter and uptake of membrane-impermeant probes.

Thus, there is both a need for discrimination of cells that were dead prior to permeabilization and the lack of suitable techniques for doing so. Physical separation methods such as percoll and ficoll density gradient separation can separate dead from live cells on the basis of differing buoyant densities (5), but have the disadvantages of wide variations in purity and recovery, selective cell loss (11), and in some circumstances, cell toxicity (21). Light scatter-based dead cell discrimination is often inapplicable to heterogeneous cell mixtures such as solid tumors, because dead cells of one type may have light scatter characteristics similar to those of live cells of another type (15,20). Lastly, few of the known fluorescent viability probes (e.g., propidium iodide, fluorescein diacetate, etc.) are considered compatible with the use of permeabilizing agents, primarily due to weak or reversible binding, allowing them

to leach out of stained cells and into artificially permeabilized unstained cells (15,20).

Pollack et al. have reported successful dead cell discrimination using PI in ethanol-fixed samples (14).

The present invention provides viability probes for inclusion in solid tumor monoclonal antibody panels, which established the following criteria: broad cell type specificity; low nonspecific staining of live cells; high signal-to-noise staining of dead cells; binding in dead cells of sufficiently high affinity to withstand the permeabilization procedure; and excitation and emission spectra compatibility with the other dyes in the solid tumor panel.

Disclosure of the Invention

Accordingly, it is an object of the subject invention to provide viability probes which are useful for inclusion in a monoclonal antibody panel for the analysis of solid, preferably breast, tumors.

It is another object of the present invention to provide a viability probe directed against a relatively stable intracellular antigen.

It is an additional object of the present invention to provide a viability probe that provides information regarding late apoptotic and necrotic cells containing degraded chromatin (Type III).

It is an additional object of the present invention to provide a viability probe that provides information regarding permeabilized cells containing native chromatin (Type I).

It is another object of the present invention to provide a viability probe that has the significant advantage of being resistant to fixation and permeabilization procedures, such as are often required for sample preservation, biohazard control, or intracellular labelling.

It is another object of the present invention to provide an antibody probe which can be conjugated to any of a wide array of available fluorophores.

The viability probes of the present invention may be use in the methods of application having docket number 139,646, entitled "Solid Tumor Analysis by Multiparametric Flow Cytometry", filed concurrently herewith. Said application is herein incorporated by reference.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows and in part will become apparent to those skilled in the art upon examination of the following or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

Brief Description of Drawings

The preferred embodiments of this invention will now be described by way of example, with reference to the drawings accompanying this specification in which:

FIG. 1 shows a sample processing flow chart;

FIG. 2 shows a comparison of light scatter (LT SCATTER) and PI staining of unprocessed cells with staining of processed cells by experimental viability probes actin-SAM-FITC (ACTIN-SF), cytokeratin-SAM-FITC (CK-SF), 7-AAD, and TO-PRO-3.

FIG.3 shows a comparison of light scatter (LT SCATTER) and PI staining of unprocessed cells (PI UNFIXED) with staining of processed cells by experimental viability probes LDS-751 (LDS), tubulin-SAM-FITC (TUBULIN-SF), EMA, and PI (PI FIX).

Modes for Carrying Out the Invention

The occurrence of often large apoptotic and necrotic populations common to solid tumors presents a significant

obstacle to the accurate flow cytometric analysis of tumor cells. Indeed, it is quite possible, particularly in large tumors or stored specimens, for live tumor cells to comprise only a small minority of the total cell population. Unless the live tumor cells can be identified and isolated, the potential for erroneous immunophenotypic and DNA analysis may be prohibitive, due to wide variations in autofluorescence background of different cell types and dead cells, nonspecific cell surface antibody binding, and nonspecific antibody uptake, and chromatin degradation in dead cells.

The present invention provides viability probes which are useful for inclusion in a monoclonal antibody panel for the analysis of solid, preferably breast, tumors.

Applicants have discovered that the viability probes of the present invention demonstrate sufficiently broad cell type specificity to be informative for the large variety of cells found in solid tumors (4); low nonspecific binding; specific binding strong enough to withstand the staining and permeabilization procedures; both high and stable fluorescence intensity, to remain informative over the period of time dead cells may persist in a solid tumor before disintegration (at which point the debris can be discriminated or gated out on the basis of light scatter); and compatibility with the four other fluorescent probes comprising the tumor antibody panel.

Applicants have found that tubulin-SAM-FITC appears to satisfy all of the aforementioned criteria: the presence of tubulin in all eukaryotic cells (18) circumvents the problem of tumor heterogeneity; the probe exhibits little or no nonspecific binding to intact cells, and its binding in permeabilized cells is sufficiently strong to withstand the staining and permeabilization procedures; its signal-to-noise ratio is high enough to clearly distinguish it from autofluorescence even through day 15 after cell harvest, and its fluorescence intensity diminishes relatively slowly over the time course; and it

is compatible with the other probes comprising the tumor antibody panel. Cytokeratin-SAM-FITC was satisfactory in every respect other than its specificity only for epithelial cells. Actin, EMA, and 7-AAD did not display sufficiently high signal-to-noise ratios over the entire time course. TO-PRO-3, LDS-751, and PI (processed cells) similarly stained both live and dead cells, and are therefore uninformative for viability in this application.

Recent reports that membrane permeabilization precedes chromatin degradation in necrosis and follows it in apoptosis (1,5,7,13) imply the occurrence of a least three significant types of dead cells, described in Table 1.

Table 1

Types, Characteristics, and Detection of Dead Cells

DEAD CELL TYPE	STAGE OF CELL DEATH	CHARACTERISTICS	VIABILITY PROBE DETECTION
I	Early necrosis	Native chromatin/ permeable cell membrane	DNA-specific dyes compatible with fixation/artificial permeabilization; anti-tubulin
II	Early apoptosis	Degraded chromatin/ intact cell membrane	None compatible with fixation/ artificial permeabilization
III	Late necrosis/ Late apoptosis	Degraded chromatin/ permeable cell membrane	anti-tubulin

The present invention supports this categorization. The existence of Types I and III dead cells is suggested by the fact that staining by DNA-specific probes is most intense at the onset of cell death and decreases with increasing cell age after harvest. The existence of Type II dead cells, with intact membranes and degraded chromatin, is predicted by the consistently higher percentages (Tables 2 and 3) of cells with low forward

scatter (suggesting the loss of cell volume that precedes chromatin degradation in apoptosis) (7) than positivity for the viability probes (excluding TO-PRO-3, LDS-751, and PI in processed cells, all of which stained live and dead cells indiscriminately).

The viability probes of the present invention are further directed against a relatively stable intracellular antigen which proves more informative than traditional, DNA-specific viability dyes (e.g., propidium iodide) for late apoptotic and necrotic cells containing degraded chromatin (Type III), as well as permeabilized cells containing native chromatin (Type I). The present invention has the significant advantage of being resistant to fixation and permeabilization procedures, such as are often required for sample preservation, biohazard control, or intracellular labelling. Finally, the present invention teaches the use of an antibody probe, which can be conjugated to any of a wide array of available fluorophores, which has broader application potential than more traditional dye probes of fixed, and frequently broad, emission spectra.

In a preferred embodiment of the present invention the viability probe is cytokeratin. In the most preferred embodiment of the present invention the viability probe is tubilin.

The present invention provides two time-course studies presented below, wherein tumor cell lines were induced to enter a condition of cellular hypoxia intended to simulate the internally hypoxic environment of a solid tumor, and in which their degree of staining by numerous viability probes is compared. The first study involved staining of the MDA (breast tumor) cell line by propidium iodide (PI) without permeabilization as the standard, and, with permeabilization, anti-actin-SAM-FITC, anti-cytokeratin-SAM-FITC, 7-aminoactinomycin D (7-AAD; a high-affinity DNA intercalator with an emission maximum at approximately 650 nm) (16,17), and TO-PRO-3 (a membrane-

impermeant, high-affinity DNA intercalator with an emission maximum at 661 nm, and minimal fluorescence except when bound to nucleic acids). The second study included staining of MDA cells by PI without permeabilization as the standard, and, with permeabilization, anti-tubulin-SAM-FITC, ethidium monoazide bromide (EMA, a photoactivated, covalently binding DNA intercalator with an emission maximum at approximately 600 nm) (15), LDS-751 (a vital nucleic acid stain with an emission maximum at 670 nm) (20), and PI.

Materials

PBS, lysophosphatidyl choline, Nonidet P-40 (NP40), trypan blue, anti-α-smooth muscle actin monoclonal antibody, and 7-aminoactinomycin D (7-AAD) were obtained from Sigma Chemical Co. (St. Louis, MO). FBS and absolute methanol were purchased from HyClone (Logan, UT) and JT Baker, Inc. (Phillipsburg, NJ), respectively. Propidium iodide (PI) stain (from the DNA-Prep Kit; contains 50 ug/ml PI, 4 KU/ml bovine pancreas Type III RNAse, 0.1% NaN_3, saline, and stabilizers) and anti-cytokeratin monoclonal antibody were provided by Coulter Corporation (Miami, Florida). Anti-α-tubulin was purchased from Zymed Laboratories, Inc. (San Francisco, CA). FITC-conjugated sheep-anti-mouse F(ab') fragments (SAM-FITC) were obtained from Silenus Laboratories (Victoria, Australia). Ethidium monoazide bromide (EMA) and TO-PRO-3 were from Molecular Probes, Inc. (Eugene, OR). LDS-751 was purchased from Exciton (Dayton, OH).

Cell Culture

Monolayer cultures of the MDA-MB-175-VII breast cancer cell line (American Type Culture Collection, Rockville, MD) were grown in high-glucose DMEM (Biowhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) at 37°C and 5% CO_2 in a humidified atmosphere. The cultures were maintained

in log phase growth prior to harvesting. For each of the time-course studies presented here, cells were harvested daily (excluding weekends) and subsequently held, tightly capped, at 37° and 5% CO_2 in a humidified atmosphere, to simulate intratumoral hypoxia. In each study, upon harvest of the twelfth daily batch of cells, samples from each batch were processed for flow cytometric analysis.

Cell Staining

For purposes of evaluating viability probes for inclusion in our solid tumor monoclonal antibody panel, samples were processed according to the procedure used in our laboratory for simultaneous surface and intranuclear staining of solid tumors.

In each of the time-course studies, after the last of twelve daily (excluding weekends) batches of cells was harvested, aliquots were taken from each batch for trypan blue viability staining and microscopic enumeration. Cells from each batch were then aliquoted, at 1 x 10^6 cells per test, into 12 x 75 mm siliconized glass test tubes. In each experiment, the samples were divided into two groups: an unprocessed (i.e., not processed by the solid tumor staining procedure below) series to be stained with propidium iodide (PI) as a standard, and a series to be stained with other viability dyes and processed according to the solid tumor staining procedure below (Fig. 1). Thus, in Study 1, for each day's harvest of MDA cells (day 0, the youngest cells, through day 15, the oldest cells), there were seven samples: an unprocessed, unstained control; an unprocessed, PI-stained standard; a processed, unstained control; and four processed samples stained with either actin-SAM-FITC, cytokeratin-SAM-FITC, 7-AAD, or TO-PRO-3. In Study 2, there were also seven samples for each day's harvest of MDA cells: an unprocessed, unstained control; an unprocessed, PI-stained standard; a processed, unstained control; and four processed samples stained with either tubulin-SAM-FITC, EMA, LDS-751, or PI. It should

be noted that unstained cells, rather than isotype controls, were used here to establish fluorescence background for antibody-labelled cells. The intensity of antibody staining inside a cell is the result of a combination of factors, including specific binding to the target antigen, nonspecific antibody binding, antibody entrapment, and nonspecific binding of the dye itself. Clearly, when the measurement of specific binding to a target antigen is the objective, isotype controls must be used to estimate the level of nonspecific fluorescence. However, when, as in this case, the objective is merely to distinguish permeabilized from intact cells by fluorescent antibody uptake, the more antibody taken up (whether specifically or nonspecifically), the better the distinction. Since the determining factor in this application was total, rather than specific, fluorescence, isotype controls were unnecessary.

After aliquoting the cells, the unprocessed series to be stained with PI and their unstained controls were centrifuged at 500 x g for 5 minutes at room temperature and decanted. The unstained controls were resuspended in PBS supplemented with 2.5% FBS (PBSF), and the PI-stained samples were resuspended in 1 ml and incubated in darkness at room temperature for twenty minutes prior to flow cytometric analysis.

The remaining samples were centrifuged as above and decanted. Unstained controls and cells to be stained with 7-AAD, TO-PRO-3, LDS-751, EMA, or PI were resuspended in 200 µl PBSF and incubated for 15 minutes at room temperature. The remaining samples were resuspended in 200 µl each of either a 1:200 dilution of actin in PBSF (protein concentration unavailable), a 1:200 dilution of tubulin in PBSF (protein concentration unavailable), or 25 µg/ml cytokeratin in PBSF (optimal doses for all three antibodies were previously established by titration; data not shown), and incubated for 15 minutes at room temperature. All samples were then washed with 2 ml PBS

each, centrifuged as above, and decanted. Unstained controls were then resuspended in 200 µl PBSF and incubated for 15 minutes at room temperature. Samples stained with actin, cytokeratin, or tubulin were resuspended in 200 µl of 48.8 µg/ml SAM-FITC in PBSF and incubated in darkness for 15 minutes at room temperature. 7-AAD-stained cells were resuspended in 1 ml/test of 25 µg/ml 7-AAD (250 µl of stock solution in 49.75 ml PBSF; stock solution is 5 mg/ml in methanol) and incubated in darkness for 15 minutes at room temperature. TO-PRO-3-stained cells were resuspended in 1 ml/test of 5 µM TO-PRO-3 in PBSF and incubated in darkness for 15 minutes at room temperature. LDS-751-stained cells were resuspended in 1 ml/test of PBSF containing 10 µl of 2 µg/ml LDS-751 (50 µl of stock solution in 4.95 ml PBSF; stock solution is 0.2 mg LDS-751/ml methanol) and incubated in darkness for 15 minutes at room temperature. EMA-stained cells were resuspended in 10 µl/test of 5 µg/ml EMA (125 µl of stock solution in 4.875 ml PBSF; stock solution is 0.2 mg EMA/ml methanol) and incubated for 15 minutes at room temperature, at a distance of 20 cm from a fluorescent light bulb. PI-stained cells were resuspended in 1 ml/test of the same PI solution used for unprocessed cells and incubated in darkness for 15 minutes at room temperature. All samples were then washed as above, followed by resuspension in 200 µl/test PBSF, and a 15-minute, room-temperature incubation in darkness (to simulate surface antigen staining of tumor samples). All samples were then washed twice more as above. After decanting the supernatant, each sample was resuspended in 1 ml of 25°C 20 µg/ml lysophosphatidyl choline in 1% paraformaldehyde, incubated for 2 minutes at room temperature, centrifuged at 500 x g for 5 minutes at room temperature, and decanted. The samples were then resuspended in 1 ml/test of -20°C absolute methanol and incubated on ice for 10 minutes, followed by centrifugation as above. After decanting, the samples

were resuspended in 1 ml/test of 4°C 0.1% NP40 and incubated on ice for 5 minutes, followed by centrifugation as above. After decanting, samples were resuspended in 200 µl/test of PBSF and incubated in darkness for 15 minutes at room temperature (to simulate intracellular antigen staining of tumor samples), followed by washing as above. Finally, the samples were resuspended in 1 ml/test PBSF prior to flow cytometric analysis.

Flow Cytometry

Samples were analyzed on an EPICS® Elite flow cytometer (Coulter Corporation, Miami, FL) configured with five photomultiplier tubes and three lasers: a water-cooled, 5 W argon laser, an air-cooled 488 nm argon laser operated at 15 mW, and a 10 mW air-cooled 633 nm helium-neon laser. TO-PRO-3 stained samples were analyzed with 633 nm excitation; all other samples were analyzed with 488 nm excitation by the air-cooled argon laser. Fluorescence emission of actin-, cytokeratin-, and tubulin-SAM-FITC was reflected by a 550 nm dichroic long-pass filter and passed through a 525 nm bandpass filter. PI and EMA fluorescence were reflected by a 650 nm dichroic long-pass filter and passed through a 610 nm bandpass filter. 7-AAD, TO-PRO-3, and LDS-751 fluorescence passed through 650 nm dichroic long-pass and 675 nm bandpass filters. Autofluorescence of unstained controls was measured through the same filter configurations as the stained samples with which they were matched.

Five thousand events were collected in listmode format for each sample, with linear amplification of forward and side scatter, and four-decade logarithmic amplification of fluorescence. Acquisition and analysis were performed with the Elite software; color graphics were performed with WinList software (Verity Software House, Topsham, Maine) and Excel® software (Microsoft Corporation, Redmond, WA).

The results of the first time-course study are presented in FIG. 2 and Table 2. In FIG. 2, the leftmost column indicates the number of days the batch of cells in the corresponding row was held after harvest (days 5, 6, 12 and 13 coincided with weekends, during which cells were not harvested). The next column displays light scatter results for unprocessed cells from each batch (in each of these histograms, forward scatter is on the Y axis, and side scatter is on the X axis). The remaining columns display fluorescence histograms of unstained cells (in green) electronically overlaid with histograms (in colors other than green) of cells stained with the probe indicated at the top of each column. All instrument settings remained unchanged for the duration of the experiment. Fluorescence histograms are on a four-decade logarithmic scale. All histograms contain 5,000 autoscaled events.

In general, dead cells exhibit a decrease in forward angle light scatter, an increase in 90° angle light scatter, and an increase in fluorescence when stained (prior to or in the absence of artificial permeabilization) with fluorescent probes (17). Thus, to satisfy our criteria, a viability probe should display, in FIGS. 2 and 3, three characteristics: 1) little or no staining of live cells (i.e., the non-green peaks of the test samples in the first row of FIGS. 2 and 3 should match the green peaks of the unstained controls they overlay as closely as possible); 2) fluorescence bimodality comparable to the proportion of live and dead cells in the sample, as indicated in FIGS. 2 and 3 by light scatter (i.e., the non-green peaks of the test samples should be bimodal in rows [primarily days 1-4] in which the light scatter histogram indicates the presence of both live and dead cells, and should be unimodal in rows [primarily days 0 and 7-15] in which the light scatter histogram indicates the presence of only live or only dead cells); and 3) fluorescent staining of dead

Table 2

Study 1: Percent Positivity and Mean Channel Fluorescence Comparison of Experimental Viability Probes

DAY	LS %POS[a]	PI (Unprocessed Cells) %POS[b]	MC[c]	ACTIN-SAM-FITC %POS	MC	CYTOKERATIN-SAM-FITC %POS	MC	7-AAD %POS	MC	TO-PRO 3 %POS	MC
0	13.2	6.8	768.4	6.6	14.4	9.1	46.3	4.2	9.86	100	264.8
1	69.5	52.3	903.2	49.8	21.4	54.2	538.9	43.9	12.4	100	154.3
2	73.4	61.3	611.4	55.8	15.2	57.4	377.3	54.1	11.0	100	144.7
3	82.8	65.7	409.8	68.1	22.0	68.8	319.9	67.8	9.44	100	119.2
4	91.8	68.3	263.6	75.5	14.1	75.5	289.7	-	3.68	100	74.9
7	100	82.3	106.1	95.6	8.04	88.0	172.9	-	3.70	100	42.4
8	100	86.7	68.6	96.2	8.85	88.3	215.9	-	3.77	100	32.8
9	100	85.3	62.2	94.7	11.4	86.2	192.6	-	3.88	100	38.0
10	100	87.3	47.3	94.5	8.58	90.1	178.5	-	2.86	100	30.7
11	100	86.2	43.3	91.0	11.9	82.4	170.7	-	3.12	100	28.6
14	100	88.8	36.7	93.2	15.7	84.7	203.6	-	4.49	100	25.3
15	100	84.7	32.3	94.4	9.83	-	95.9	-	3.26	100	20.4

[a] LS %POS refers to the percentage of cells displaying the low forward scatter characteristic of dead cells

[b] %POS refers to percent positivity

[c] MC refers to mean channel (unconverted values from a 4-decade logarithmic scale)

cells that is significantly brighter than the autofluorescence of the unstained controls (i.e., the positive non-green peaks of the test samples should be as far apart as possible from the green peaks of the unstained controls they overlay). It should be emphasized that the green peaks in each fluorescence histogram correspond to unstained controls (electronically overlaid with stained samples in colors other than green), not negatively stained cells.

In FIG. 2, nonspecific staining of live cells was minimal for actin-SAM-FITC and cytokeratin-SAM-FITC, dim for 7-AAD, relatively bright for PI, and very bright for TO-PRO-3. The fluorescence distributions of PI, actin-SAM-FITC, cytokeratin-SAM-FITC, and 7-AAD corresponded to light scatter and trypan blue uptake (data not shown) in the relative proportions of their negative (live cell) and positive (dead cell) peaks; TO-PRO-3's fluorescence distribution remained unimodal throughout the time course. Of the three probes -- actin-SAM-FITC, cytokeratin-SAM-FITC, and 7-AAD -- suitable for this application, cytokeratin-SAM-FITC exhibits the highest signal-to-noise ratio, remaining distinct from autofluorescence through day 15. By day 4, neither actin nor 7-AAD staining is significantly distinct from autofluorescence. Neither PI, due to its reversible binding, nor TO-PRO-3, because of its intense staining of both live and dead cells without significant distinction, are acceptable as viability probes for this application.

In Table 2, the percentages of cells stained positively by the various viability probes used in Study 1 are compared with the percentages of cells displaying the low forward and high side scatter characteristic of dead cells. Because certain probes stained live cells nonspecifically, percentage positivity of staining was calculated relative to the negative peak in bimodal distributions, rather than to unstained cells. At the point in each column where bimodality was completely lost,

a dash appears in the table. Interestingly, the light scatter-based percentages are consistently and significantly higher than the fluorescence-based percentages. PI, actin-SAM-FITC, cytokeratin-SAM-FITC, and 7-AAD yield comparable positivity through day 4, when the bimodality of the 7-AAD distribution is lost. PI, actin, and cytokeratin positivity remain comparable through day 15 after harvest. This finding is significant, because it suggests that the much greater size of the antibody sandwich complexes, relative to the DNA-specific dyes, does not impede staining. The unimodal distribution of TO-PRO-3 throughout the study, and its intense staining of live cells, make it uninformative here for viability. Mean channel values of fluorescence intensity are also reported. In general, after day 0, when staining is minimal (except for TO-PRO-3) due to the preponderance of live cells, the fluorescence intensity of all the probes decreases, though at different rates. PI and TO-PRO-3 fluorescence intensities show the steepest decline, followed by cytokeratin and, in tandem, actin and 7-AAD. Conversely, autofluorescence (green histograms) increases steadily, particularly in the green region of the spectrum, with cell age after harvest.

The results of the second time-course study are presented in FIG. 3 and Table 3. The formats of both are the same as those of FIG. 2 and Table 2, respectively. The light scatter, autofluorescence, and PI staining of unprocessed cells are similar to those in FIG. 2. In FIG. 3, nonspecific staining of live cells was minimal for tubulin-SAM-FITC, and relatively high for PI (in both processed and unprocessed cells), LDS-751, and EMA. The fluorescence distributions of PI in unprocessed cells, tubulin-SAM-FITC, and EMA corresponded to light scatter and trypan blue uptake in the relative proportions of their negative and positive peaks; the fluorescence distributions of PI in processed cells and LDS-751

Table 3

Study 2: Percent Positivity and Mean Channel Fluorescence Comparison of Experimental Viability Probes

DAY	LS %POS[a]	PI (Unprocessed Cells)		LDS-751		TUBULIN-SAM-FITC		EMA		PI (Processed Cells)	
		%POS[b]	MC[c]	%POS	MC	%POS	MC	%POS	MC	%POS	MC
0	14.0	5.9	483.0	98.2	12.4	6.2	10.3	4.2	12.4	100.0	6.42
1	59.8	40.7	547.2	97.7	31.2	36.6	273.1	31.6	19.7	97.1	29.8
2	76.7	51.0	358.4	93.7	20.2	56.9	383.2	49.9	16.7	93.3	31.7
3	85.2	66.2	242.1	87.2	21.4	67.1	330.3	58.6	15.9	91.0	27.6
4	88.7	70.6	155.8	88.4	53.5	74.7	339.1	71.6	16.6	93.1	10.8
7	100	87.4	67.1	88.8	72.0	81.8	323.8	84.4	13.2	92.5	4.12
8	100	86.8	41.0	84.1	66.5	82.4	343.9	86.6	16.9	90.3	3.77
9	100	89.4	31.5	95.3	40.5	86.4	328.3	87.9	14.8	96.0	3.63
10	100	89.3	51.5	90.8	66.9	89.2	205.2	88.8	13.5	94.4	5.08
11	100	89.8	25.3	86.7	68.0	90.0	188.2	90.1	12.0	91.3	3.70
14	100	89.4	15.8	89.5	61.2	85.0	272.2	90.2	12.4	91.5	4.02
15	100	88.2	15.1	83.9	58.4	88.6	284.0	90.2	12.8	91.9	4.43

[a] LS %POS refers to the percentage of cells displaying the low forward scatter characteristic of dead cells

[b] %POS refers to percent positivity

[c] MC refers to mean channel (unconverted values from a 4-decade logarithmic scale)

remained essentially unimodal throughout the time course. Of the two probes -- tubulin-SAM-FITC and EMA -- suitable for this application, tubulin-SAM-FITC exhibits the highest signal-to-noise ratio, remaining very distinct from unstained cells through day 15. By day 7, the difference between EMA staining and autofluorescence is approximately the same as the level of nonspecific staining in live cells at day 0. Neither PI, due to its reversible binding, nor LDS-751, because of its consistently unimodal fluorescence distribution, are acceptable as viability probes for this application.

As in Table 2, the light scatter-based percentages of dead cells in Table 3 are consistently and significantly higher than the fluorescence-based percentages. PI in unprocessed cells, tubulin-SAM-FITC, and EMA yield comparable positivity throughout the time course. The consistently unimodal distributions of LDS-751 and PI in processed cells make them uninformative here for viability. The mean fluorescence intensity of PI in both processed and unprocessed samples declines rapidly over the time course, while that of EMA and tubulin-SAM-FITC diminishes slowly. Interestingly, the fluorescence intensity of LDS-751 appears to be cyclical over the time course, while all of the other probes exhibit a linear decrease in fluorescence intensity.

All publications cited in this specification are indicative of the level of skill of those in the art to which this application pertains. Each publication is individually incorporated herein by reference in the location where it is cited.

One skilled in the art will appreciate that although specific reagents and conditions are outlined in the above preparations and methods, modifications can be made which are meant to be encompassed by the spirit and scope of the invention. The preparations and methods, therefore, are provided to illustrate the invention. Such alternate means are to be construed as included within the intent

and spirit of the present invention as defined by the following claims.

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Claims

1. A method of discriminating dead cells in a heterogeneous biological sample which method is characterized by:

(a) staining cells with a viability probe said probe being resistant to fixation and permeabilization procedures; and

(b) analyzing by a fluorescence measuring instrument.

2. The method according to claim 1 further characterized by the viability probe which is a nucleic acid specific dye.

3. The method according to claim 1 further characterized by the viability probe which is an intracellular antigen.

4. The method according to claim 3 further characterized by the intracellular antigen which is tubulin.

5. The method according to claim 1 further characterized by the viability probe which is a cytoskeletal antigen.

6. The method according to claim 5 further characterized by the cytoskeletal antigen which is cytokeratin.

7. The method of claim 1 further characterized by the cells which are neoplastic cells.

8. The method of claim 7 further characterized by the neoplastic cells which are solid tumor cells.

9. The method of claim 7 further characterized by the neoplastic cells which are breast tumor cells.

10. The method of claim 1 further characterized by the cells which are non-adherent tumor cells.

11. The method of claim 10 further characterized by the non-adherent tumor cells which are hematopoietic neoplasias.

12. A method of discriminating late apoptotic cells containing degraded chromatin (Type III) which method is characterized by:

(a) staining cells with a viability probe, said probe being resistant to fixation and permeabilization procedures; and

(b) analyzing by flow cytometry.

13. A method of discriminating necrotic cells containing degraded chromatin (Type III) which method is characterized by:

(a) staining cells with a viability probe, said probe being resistant to fixation and permeabilization procedures; and

(b) analyzing by flow cytometry.

14. A method of discriminating permeabilized cells containing native chromatin (Type I) which method is characterized by:

(a) staining cells with a viability probe, said probe being resistant to fixation and permeabilization procedures; and

(b) analyzing by flow cytometry.

15. A method of discriminating dead cells in a heterogeneous biological sample which method is characterized by:

(a) staining cells with a viability probe, said probe being resistant to fixation and permeabilization procedures;

(b) staining with an antibody probe; and

(c) analyzing by a fluorescence measuring instrument.

16. The method according to claim 15 further characterized by the viability probe which is a nucleic acid specific dye.

17. The method according to claim 15 further characterized by the viability probe which is an intracellular antigen.

18. The method according to claim 17 further characterized by the intracellular antigen which is tubulin.

19. The method according to claim 15 further characterized by the viability probe which is a cytoskeletal antigen.

20. The method according to claim 19 further characterized by the cytoskeletal antigen which is cytokeratin.

21. The method of claim 15 further characterized by the cells which are neoplastic cells.

22. The method of claim 21 further characterized by the neoplastic cells which are solid tumor cells.

23. The method of claim 21 further characterized by the neoplastic cells which are breast tumor cells.

24. The method of claim 15 further characterized by the cells which are non-adherent tumor cells.

25. The method of claim 24 further characterized by the non-adherent tumor cells which are hematopoietic neoplasias.

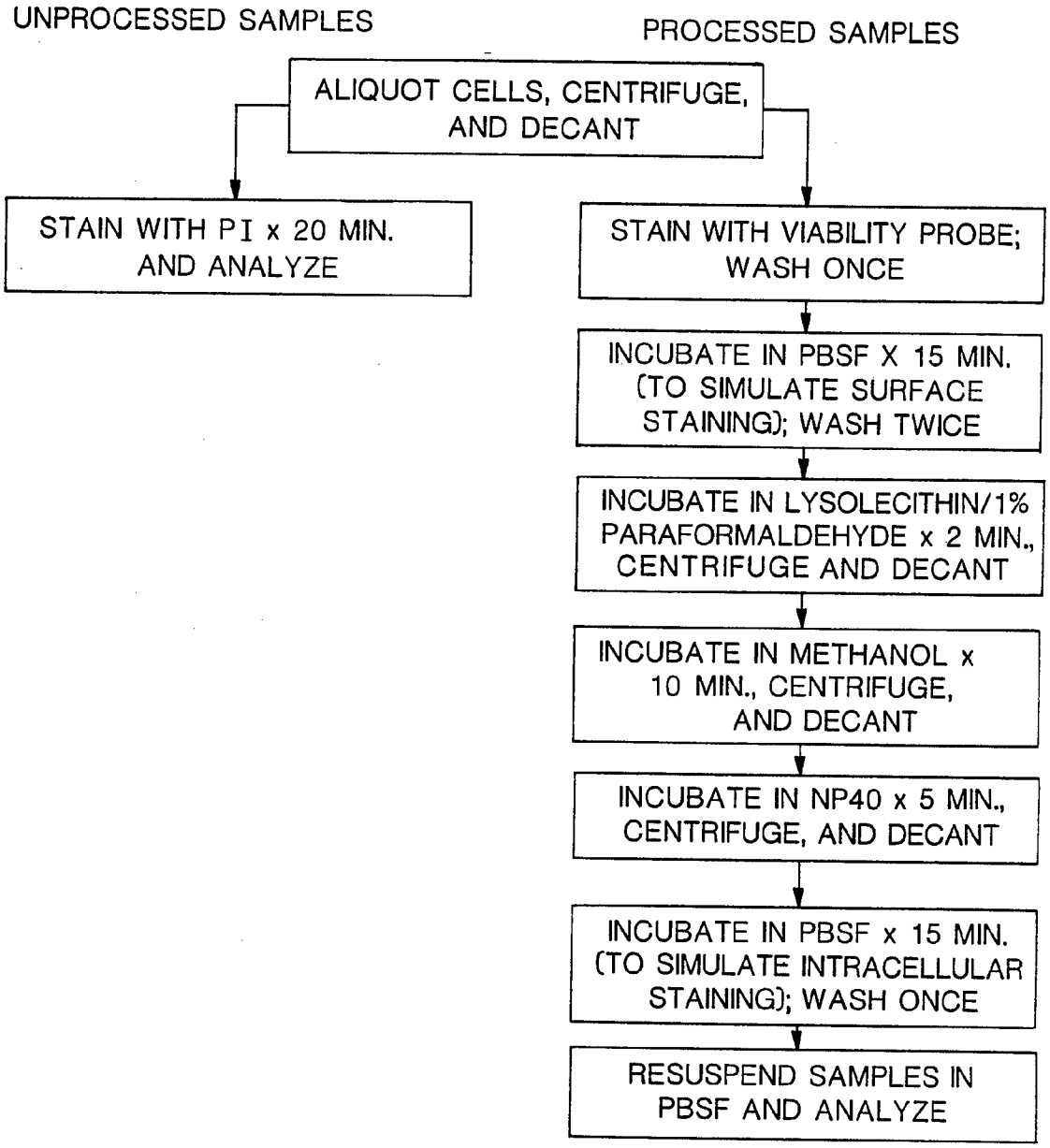
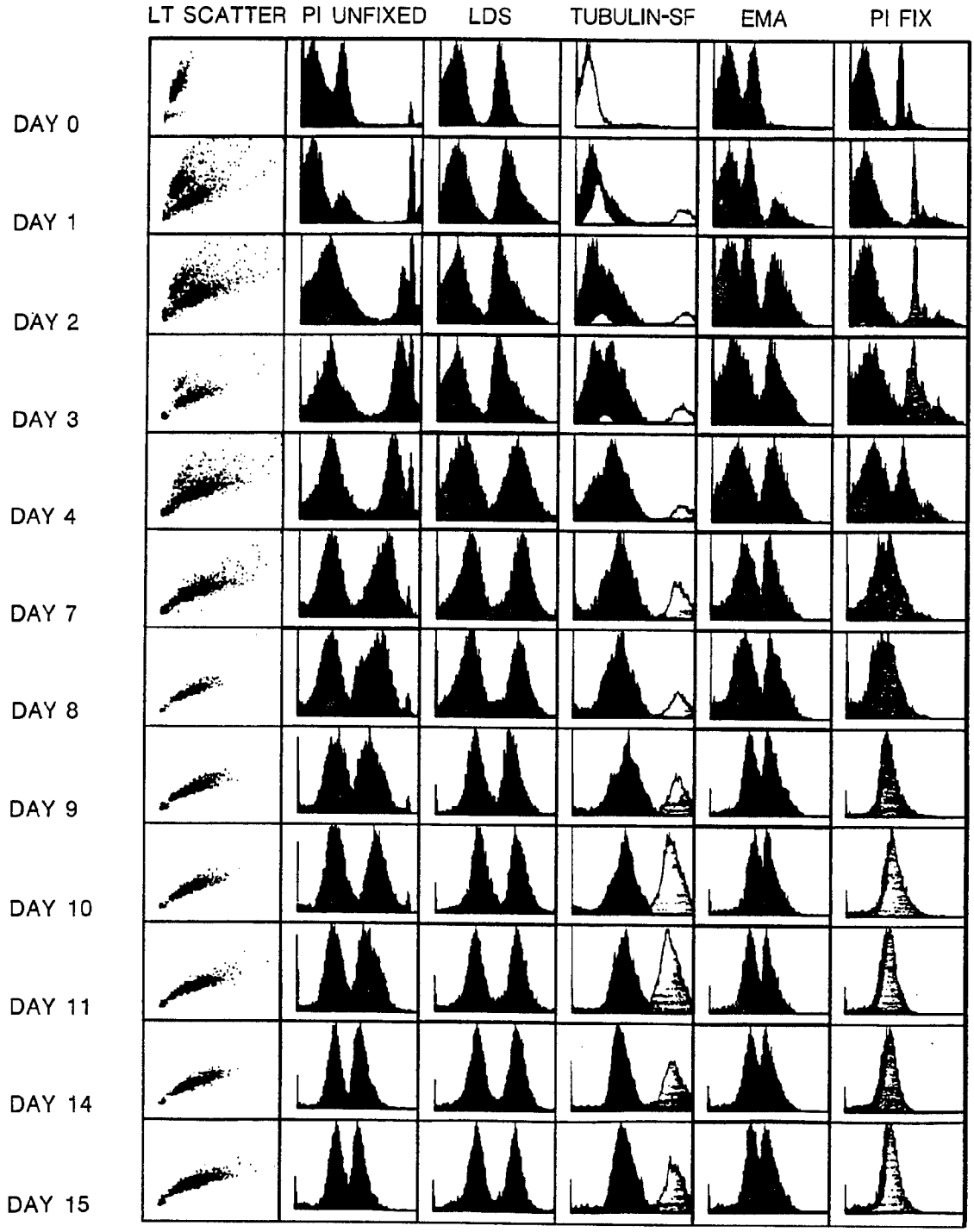

UNPROCESSED SAMPLES
PROCESSED SAMPLES
ALIQUOT CELLS, CENTRIFUGE, AND DECANT
STAIN WITH PI x 20 MIN. AND ANALYZE
STAIN WITH VIABILITY PROBE; WASH ONCE
INCUBATE IN PBSF X 15 MIN. (TO SIMULATE SURFACE STAINING); WASH TWICE
INCUBATE IN LYSOLECITHIN/1% PARAFORMALDEHYDE x 2 MIN., CENTRIFUGE AND DECANT
INCUBATE IN METHANOL x 10 MIN., CENTRIFUGE, AND DECANT
INCUBATE IN NP40 x 5 MIN., CENTRIFUGE, AND DECANT
INCUBATE IN PBSF x 15 MIN. (TO SIMULATE INTRACELLULAR STAINING); WASH ONCE
RESUSPEND SAMPLES IN PBSF AND ANALYZE

FIG. 1


LT SCATTER
PI UNFIXED
LDS
TUBULIN-SF
EMA
PI FIX
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DAY 1
DAY 2
DAY 3
DAY 4
DAY 7
DAY 8
DAY 9
DAY 10
DAY 11
DAY 14
DAY 15

FIG. 3

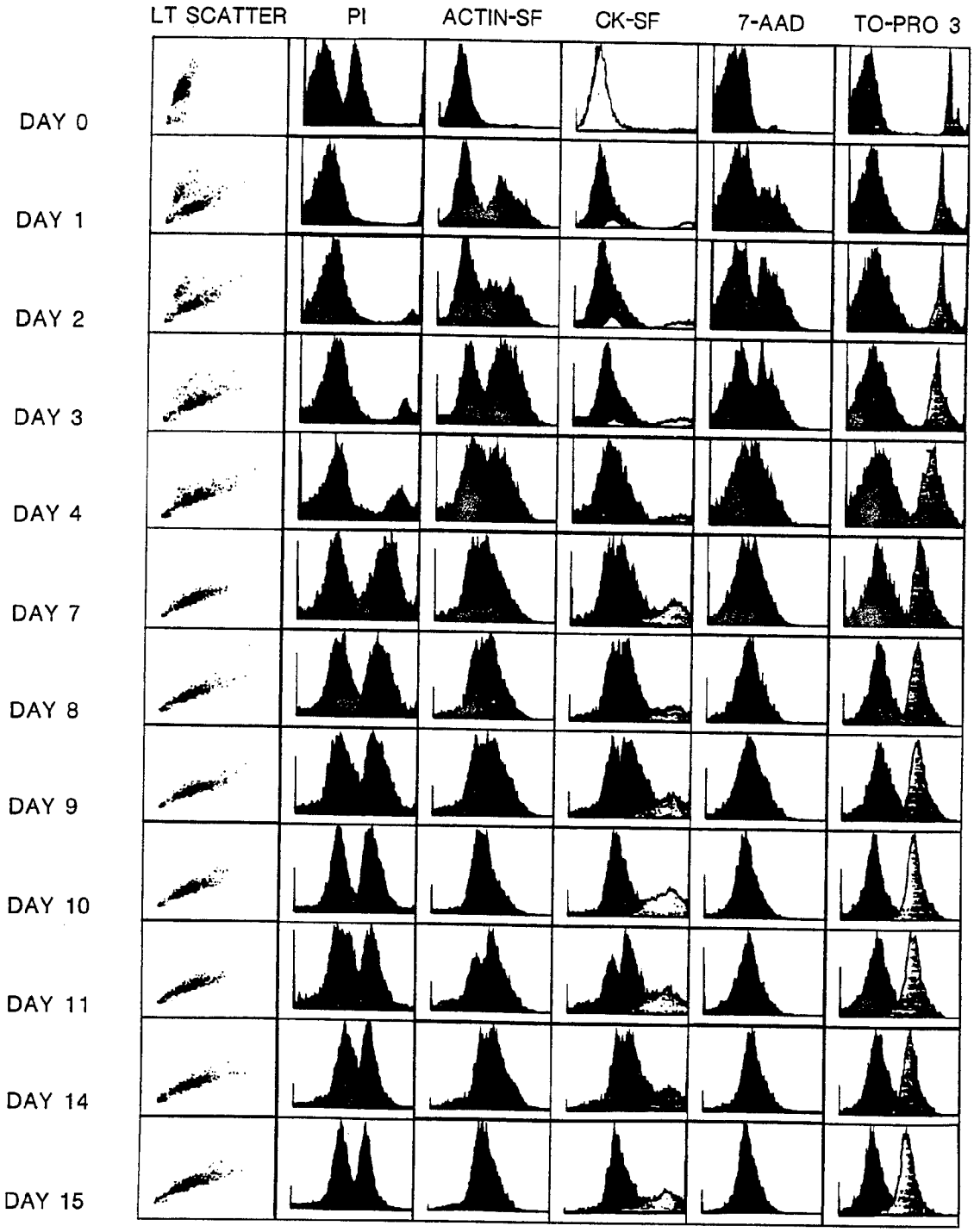
LT SCATTER
PI
ACTIN-SF
CK-SF
7-AAD
TO-PRO 3
DAY 0
DAY 1
DAY 2
DAY 3
DAY 4
DAY 7
DAY 8
DAY 9
DAY 10
DAY 11
DAY 14
DAY 15

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00513

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 21/64, 33/49, 33/533, 33/536, 33/574
US CL :435/2, 6, 7.23, 7.24; 436/64, 172, 546
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 6, 7.23, 7.24; 436/64, 172, 546

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE
search terms: viability, dead, tubulin, cytokeratin, neoplastic, breast, cancer, flow cytometry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Cytometry, Volume 9, issued 1988, L.W.M.M. Terstappen et al, "Discriminating Between Damaged and Intact Cells in Fixed Flow Cytometric Samples", pages 477-484, see entire document.	1, 2, 12-16 ---------- 3-11, 17-25
X ---- Y	Cytometry, Volume 12, issued 1991, M.C. Riedy, et al, "Use of a Photolabeling Technique to Identify Nonviable Cells in Fixed Homologous or Heterologous Cell Populations", pages 133-139, see entire document.	1, 2, 12-16 ---------- 3-11, 17-25
Y	K. D. Bauer et al, "Clinical Flow Cytometry. Principles and Application", published 1993 by Williams and Wilkins (Baltimore, MD), pages 157-175, see pages 167-172.	1-9, 12-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search
17 APRIL 1995

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00513

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochimica et Biophysica Acta, Volume 1133, issued 1992, C. Dive et al, "Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry", pages 275-285, see entire document.	10, 11, 24, 25